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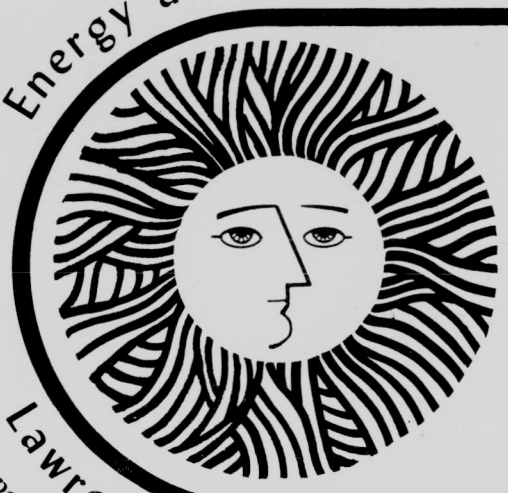

Conformational Changes In Bacteriorhodopsin Accompanying Ionophore Activity

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CONFORMATIONAL CHANGES IN BACTERIORHODOPSIN ACCOMPANYING IONOPHORE ACTIVITY

by

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Bacteriorhodopsin is at present one of the best examples in biology of an ion pump (1) and much interest exists in its characterization because the question of how hydrogen and electron carriers function as ionophores is one of the main unsolved problems of bioenergetics. It has been pointed out by Kagawa in this symposium (2) that there is evidence of the presence of a proton pump in mitochondrial complexes like ATPase, and other redox components like the b-c_1 complex, and cytochrome oxidase also exhibit proton translocation activity and by analogy may be a model for other membrane associated energy transductions.

Two types of studies will be reported upon here which are relevant to BR as an ionophore.

I. Factors involved in orienting bacteriorhodopsin in planar membranes and bacteriorhodopsin in liposomes (BR-liposomes) and how this affects photo-induced H^+ gradients and electrical potentials.

II. Chemical modification studies of bacteriorhodopsin with cross-linking reagents which provide evidence of BR structure important for its stability and that protein conformational changes are involved in ionophore activity.

Bacteriorhodopsin in Liposomes and Planar Membranes

H^+ Pump Activity in BR-Liposomes

To study the orientation of BR in purple membrane fragments we have incorporated it into egg lecithin liposomes by sonication in the presence of various salt solutions. If prepared in the presence of KCl, BR-liposomes show maximum proton pump activity when illuminated

in the presence of 100mM KCl. This is the standard BR-liposome system previously studied by Racker (3). Some variations in activity of reconstituted systems may be expected because the activity varies with the nature of the lipid used, the lipid to protein ratio, the sonication time used in vesicle formation, pH of measurement, etc. The basic activity in our reconstituted system (rate and extent of proton translocation activity 119 ng H^+/mg protein/min and 27 ng H^+/mg protein, respectively) compares favorably with other studies.

In experiments with other ions we found that increasing amounts of externally added Ca^{2+} remarkably caused an eight-fold increase in the proton pumping activity (Fig. 1). What is the nature of the effect of the cations? The specificity of the cation effects was further studied and it was observed that other ions were also effective, with bivalent ions being generally more stimulatory than monovalent. Calcium was the best of a series of bivalent cations studied. In the presence of BR-liposomes prepared in the presence of KCl, presumably K^+ is the counter ion for the proton. Indeed if valinomycin is added to overcome a possible permeability barrier to K^+ we observed, as expected, a stimulation of proton pump activity. However, a further enhancement of this activity is still given by Ca^{2+}, with the stimulation being almost additive. It may be concluded that the effect of the Ca^{2+} is not on liposome permeability <u>per se</u> but is some other effect.

Possible explanations are that cations may a) affect the proper orientation of purple membrane fragments in the lipid membrane, b) have some direct effect on the purple membrane like reacting with fragments oriented in one but not another orientation (Skulachev, personal communication), or c) may cause membrane fusion thus increasing the total internal value per surface area of liposomes. Among these explanations b) seems most likely. Explanation a) is unlikely because the purple membrane fragments are large sheets (ca. 800-1500 Å in diameter) and on thermodynamic grounds would

be unlikely to reorient after the membrane is formed. Possibility c) is unlikely because the Ca^{++} effect is reversed if the liposomes are passed through a sephadex column to remove the cations, a procedure that would be unlikely to reverse vesicle fusion.

Photoelectrical Potentials Across Planar Membranes Plus Liposomes

It is known that lipid membranes containing chlorophyll are photosensitive (cf. 4). If such membranes are separated by solutions of different redox potential, upon illumination a charge movement and generation of a photopotential can be recorded.

To measure photopotentials we incorporated purple membrane fragments into a black lipid planar membrane system composed of oxidized cholesterol-decane since such planar membranes are generally quite fragile, we tried to improve their stability by incorporating crosslinked polymers into the membrane-forming solution. The electrical properties of the planar membrane systems studied are shown in Table I (cf. 5). When BR is incorporated, the membrane lifetime is shortened and resistance across the membrane decreases. When a membrane is formed in the presence of polystyrene membrane resistance goes up slightly. Again when BR is incorporated the membrane resistance falls slightly. The major improvement of the polymer-stabilized membrane is the considerably longer stability with time, indicated as 3-5 hr in the dark, and in some instances stability has been seen for days, either in the absence or presence of BR. Also similar results have been obtained with other polymers like polyacrylamide or collagen. Polymer incorporated membranes are also more stable to applied

electric fields. The membranes chosen for further study had a concentration of polystyrene (100 mg/ml) in the membrane-forming solution, so that the membranes were still black. Planar membranes and liposomes were studied in several different conditions; results are summarized in Figure 2. The test system had two-compartments, the inner compartment made of a teflon cup possessed a 1mm circular orifice across which the planar membrane is formed.

In the first case BR was incorporated into the planar membrane. Under these circumstances, probably because of the way in which the membrane is painted over the hole in the teflon cup, causing slight potential differences between the two compartments, some asymmetry must arise in the orientation of purple membrane fragments in the planar membrane. Thus when the light is turned on a small photopotential develops in several seconds, which decays when the light is switched off. If $CaCl_2$ is added to the inner aqueous compartments, the magnitude of this photopotential is about 80 percent larger. In this case the calcium affect may be due to interaction with BR molecules in those purple membranes fragments oriented opposite to the direction of H^+ charge transfer (cf. below).

In the second case, a membrane was formed without BR, and then purple membrane fragments added in one of the aqueous compartments. When the light is turned on there is a slow photoresponse occurring on the order of minutes. In order for a potential to be recorded, BR fragments in suspension must become oriented at the membrane interface. The slow photopotential response would therefore

seem due to the time required for the fragments in suspension to orient at the interface. Using this system we tested the effect of a dark applied membrane potential upon the photopotential. It was possible to vary the applied field over a wide range because of the increased stability of the polymer incorporated membranes. With large dark membrane potentials a photoresponses as high as 500mV, in a direction opposite to the applied field were obtained. The photoresponse over the whole range of dark potentials was linear, with a 20mV photoresponse recorded at zero applied field (Figure 3).

In the third case, a planar membrane was made without BR, and a BR-liposomes suspension added to one compartment as depicted in Figure 2. This system was first introduced by Skulachev's laboratory (6) where a transient photopotential of 150mV was reported. We find similar results but in our case the photopotential is about 220mV which spontaneously decays to a lower steady level; when the light is turned off the signal returns to the original dark level. Development of the photopotential requires the presence of $CaCl_2$ in the same aqueous compartment as the BR-liposomes. The Russian workers suggested (6) that Ca^{2+} was needed to promote interaction of liposomes with the planar membrane. In agreement we find that about one hour preincubation in the dark must be allowed before maximum photopotential development occurs, which seems consistent with the time required for the liposomes to interact with the planar membrane. The nature of this interaction requires further study. It is not known whether fusion occurs although studies of Moore (7) suggest this occurs between lecithin

liposomes and planar lipid membranes.

In the fourth case, we have examined a system in which BR is in the planar membrane, and BR-liposomes are also added to one compartment (Figure 2). If BR-liposomes are on the same side of the membrane (as in the third case described above) then we observe again a transient photopotential, but the transient potential decays all the way back to the original level even though the preparation is continuously illuminated. The transient nature of the photoresponse would seem to be explained by H^+ gradients forming both across the planar membrane (rapid response) and, partially collapse of the H^+ gradient across the liposome membrane as indicated in the diagram in Figure 2. However, if BR-liposomes are located in the outer compartment then a saturated photopotential is obtained. In this case BR in the planar membrane and in BR-liposomes is assumed to be oriented in the same direction thus sustaining the H^+ gradient.

It should be possible to use the combination of a BR-liposome system with a BR-planar membrane to investigate BR orientation, and to arrange conditions to optimize the system either for photopotential development or charge transfer.

Chemical Modification of Bacteriorhodopsin

Much attention has been focused on the fact that the highly aggregated patch of BR in the purple membrane is a stable configuration in which considerable rigidity exists (8). It was of interest to determine if by chemically crosslinking BR in the purple membrane with aldehydes and other crosslinking reagents, the inherent stability of BR could be enhanced.

Thermal Stability

We examined thermal stability and bleaching of BR. Purple membranes were heat treated at 50°C and 80°C: treatment at 80°C but not at 50°C caused bleaching (Table II). Then fragments were incorporated into liposomes to determine if they still retain proton pump activity. At both 50°C and 80°C there was some decrease in proton pump activity. Decrease of proton pump activity was consistent with the bleaching of chromophore in the 80°C treated BR. Table II shows that after chemical modification with aldehydes, the temperature induced bleaching of BR is inhibited to a considerable degree. Apparently crosslinking can improve the thermal stability of BR in the purple membrane. Two other bifunctional crosslinking reagents dimethyladipimidate (DMA) and dimethylsuberimidate (DMS) were also studied because they exist with precise chain lengths and exhibit greater specificity than aldehydes for reaction. Imidoesters react exclusively with epsilon amino groups under mildly alkaline conditions whereas glutaraldehyde reacts less specifically. Aldehydes also tend to polymerize. As shown in Table II DMA and DMS both increase thermal stability of BR.

Evidence of Conformational Changes in BR

The enhanced thermal stability of BR suggested that chemical modification might be a useful approach to seek evidence of protein conformational changes involved in proton pump activity. Thus far no structural information relevant to proton ionophore activity of BR has been obtained from spectrophotometric studies (9,10).

Figure 4 shows that treatment of purple membrane fragments with different concentrations of glutaraldehyde in the light-

or dark-adapted state, effects the proton pump activity of BR-liposomes differently. Dark adapted purple membranes were considerably more sensitive to being inactivated by the treatment with glutaraldehyde. Table III shows that DMA, which is of comparable chain length (8.5 Å) to glutaraldehyde (7.5 Å), also showed marked differences in its effect on proton pump activity when dark and light treated samples are compared. There was no difference in the activity between light- and dark-treated samples by the longer chain length DMS (11.5 Å). There was no direct relationship between the extent of amidination of light- or dark-treated samples with glutaraldehyde or DMA to the losses of proton pump activity. In support of this the monofunctional reagent ethylacetimidate (EA) which does not crosslink, amidinated extensively but there was no significant loss of proton pump activity. Tryptophanyl residues decreased significantly after chemical modification and seemed more significant than lysine residues for proton pump activity. There are 4 tryptophans and 7 lysines in the BR molecule (11). The results with glutaraldehyde (1mM treatment) also show that appreciable modification of these groups occur: 62% as compared to 39%; 72% as compared to 44%, for lysine and tryptophan groups remaining after modification respectively, for preparations treated in the dark or under illumination respectively. No such light-dark differences in chemical modification occurred when the longer chain length molecule DMS was used.

The shorter chain length molecules may permeate into the interior of the molecule and cause the crosslinking of the group present

in the interior of the molecule, but the longer chain length molecules may crosslink outside of the molecule. It will be recalled that all three crosslinking reagents were capable of conferring increased thermal stability yet they act differently on the proton pump activity.

Our results suggest that the conformational change between dark and light adapted BR is small and may occur in the interior of the molecule. The latter suggestion is based on the observation that the monofunctional EA and longer chain DMS molecule did not inhibit the proton pump but shorter crosslinking reagents showed marked differences in H^+ pump activity in dark vs. light treated samples. These observations of the effects of crosslinking reagents on dark and light adapted BR indicate that the protons may be transferred through a channel or a pore.

Summary

We conclude from planar membrane and liposome studies with bacteriorhodopsin in purple membrane fragments that:

1. the activity of bacteriorhodopsin in the purple membrane is increased, as judged by greater photopotentials across planar membranes or proton translocation in liposomes, by cations particularly bivalent cations as Ca^{++}, and by applied electric fields. The effect of bivalent ions appears to be two-fold, a direct effect on BR oriented in different directions and promoting interaction of liposomes at the planar membrane interface. Applied electrical fields appear to affect orientation of purple membranes at the planar membrane interface.

2. polymer stabilized black lipid membranes will prove quite useful for extending this test system for the study of ionophore activity of biological catalysts.
3. bacteriorhodopsin in the purple membrane patch exhibits considerable thermal stability and this intrinsic stability is enhanced by treatment with bifunctional crosslinking reagents.
4. After chemical modification of bacteriorhodopsin in the light- or dark-adapted state, differences in proton pump activity are found in BR liposomes (which are consistent with differences in the number of remaining free lysine and tryptophan residues). These results indicate that two conformational states of the protein of bacteriorhodopsin are involved in its functioning as an ionophore.
5. studies with bifunctional crosslinking reagents having a defined chain length, indicate that the proton is transferred through a channel and/or pore present in the interior of the bacteriorhodopsin molecule rather than by a translocation mechanism.

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Legends To Figures

Figure 1: Ca^{2+} stimulation of proton pump activity in bacteriorhodopsin liposomes. Liposomes were prepared by sonication in 100 mM KCl, 4.8 mg egg lecithin, and 1.7 mg BR protein/ml. Light driven H^+ pump activity was determined in the presence of various concentrations of Ca^{2+} added as the chloride salt. -O-: rate; --Δ--: extent.

Figure 2: Photoelectrical potentials of bacteriorhodopsin developed across planar membranes plus liposomes.

Figure 3: Relation between applied electrical field and photopotential development of purple membrane fragments across a polymer stabilized planar membrane.

Figure 4: Effect of glutaraldehyde treatment of purple membrane fragments in the light or dark on proton pump activity in bacteriorhodopsin liposomes. Purple membrane adapted in the light and dark were treated with the concentrations of glutaraldehyde indicated. Light driven H^+-pump activity was determined after incorporation of the glutaraldehyde-treated samples into lecithin liposomes.

TABLE I, Packer et al

EFFECT OF BACTERIORHODOPSIN AND POLYSTYRENE ON THE STABILITY OF A BLACK LIPID PLANAR MEMBRANE

Planar membrane	Membrane resistance (ohm/cm^2)	Lifetime		Breakdown voltage (applied volts)
		Dark (hours)	Illuminated (hours)	
Control[a]	$5-8 \times 10^6$	0.50	0.16–0.31	0.20–0.25
BR[b]	$5-6 \times 10^5$	0.16	0.10–0.20	0.15–0.20
Polystyrene[c]	$5-6 \times 10^7$	3–5	0.31–0.80	1.50–2.10
BR + polystyrene	$2-4 \times 10^6$	3–5	0.30– 0.80	1.30–1.80

[a] 0.2 mg oxidized cholesterol/ml octane.

[b] 5mg BR (as purple membrane fragments)/ml added to a.

[c] 100 mg/ml added to a.

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TABLE II

EFFECT OF CHEMICAL MODIFICATION ON BLEACHING AND THERMAL STABILITY ON BACTERIORHODOPSIN

	Absorbance Decrease at 570 nm (percent of zero time control)	
Treatment	50°C	80°C
None	0.1	65.5
Formaldehyde		
10mM	1.3	24.5
100mM	0.0	18.4
Glutaraldehyde		
5mM	9.7	16.0
50mM	8.5	17.3
DMA		
1.5mM	10.5	51.2
5. mM	8.9	22.8
DMS		
10mM	11.0	21.5

Purple membrane preparations were incubated at the temperatures indicated for 80 min. in 100mM KCl (pH 6.0). Absorbance at 570nm was determined as an indication of the retention of chromophore structure.

TABLE III

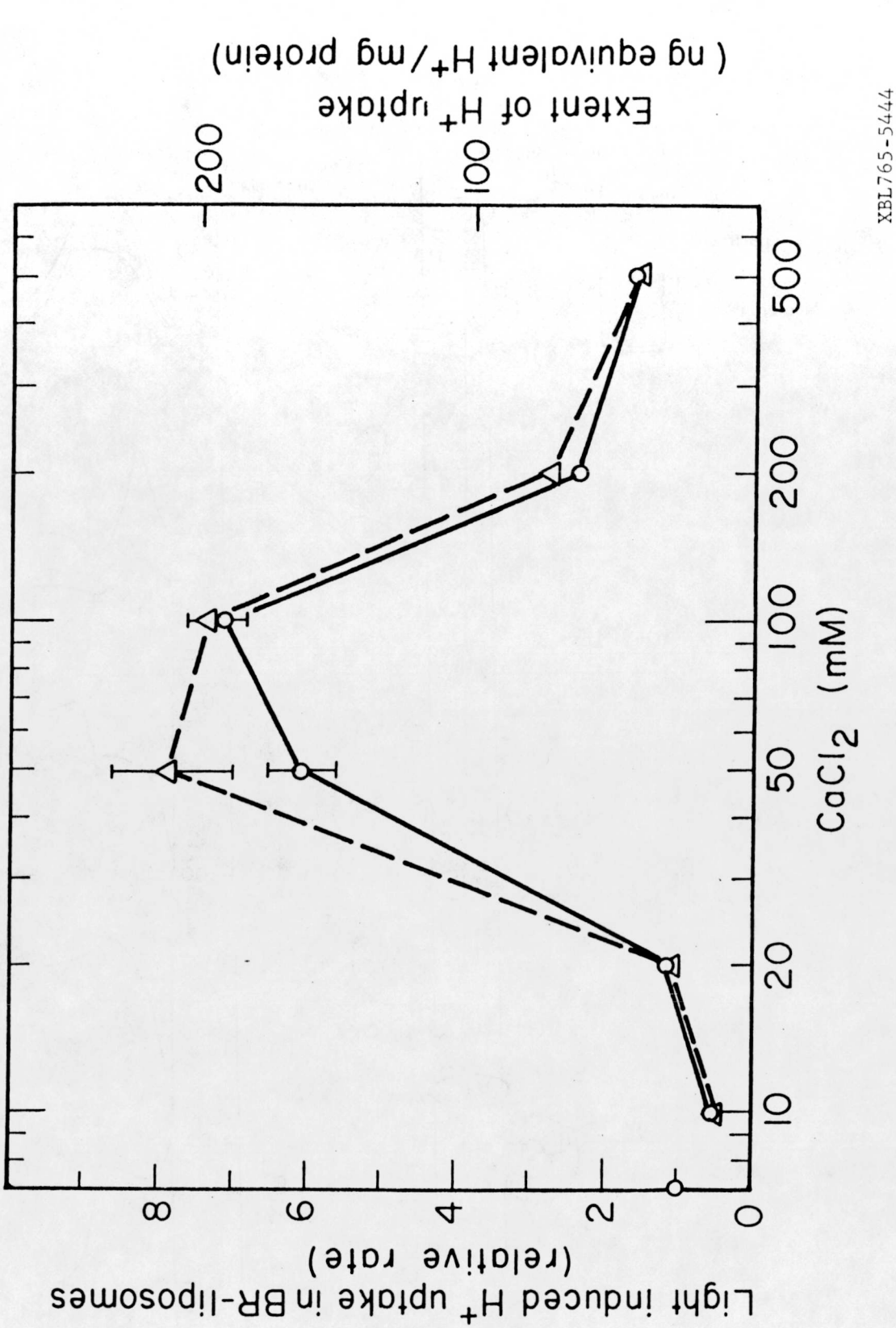
CHEMICAL MODIFICATION OF THE PURPLE MEMBRANE BY IMIDOESTERS

	H^+ Pump (ng equiv/ mg protein/min.)	Relative Activity (Percent)	Free Amino Group (Percent)	Tryptophan Residues (Percent)
EA (20 mM)[a]				
Control	73.2	100	100. (7)	100. (4)
Dark	74.6	102	58.4(4)	90.8(4)
Illuminated	90.8	124	39.2(3)	97.7(4)
DMA (10mM)[b]				
Control	333.8	100	100. (7)	100. (4)
Dark	269.3	81	40.7(3)	55.6(2)
Illuminated	456.8	137	57.7(4)	68.8(3)
DMS (10mM)[a]				
Control	56.6	100	100. (7)	100. (4)
Dark	124.1	219	64.2(4)	78.6(3)
Illuminated	120.8	213	61.9(4)	75.6(3)

After chemical modification, purple membrane fragments (300 or 500 µg protein) were incorporated into lecithin liposomes (5 mg lipid/ml) by sonication. The external pH change upon illumination (2.5×10^5 erg/cm^2/sec) were determined with a glass electrode. Free amino residues were determined fluorometrically with fluorescamine (390nm/480nm). Tryptophanyl residues were determined from intrinsic fluorescence (285nm/335nm).

[a] Egg lecithin liposomes

[b] Soybean lecithin liposomes


Light induced H+ uptake in BR-liposomes
(relative rate)
0
2
4
6
8
10
20
50
100
200
500
CaCl2 (mM)
100
200
Extent of H+ uptake
(ng equivalent H+/mg protein)

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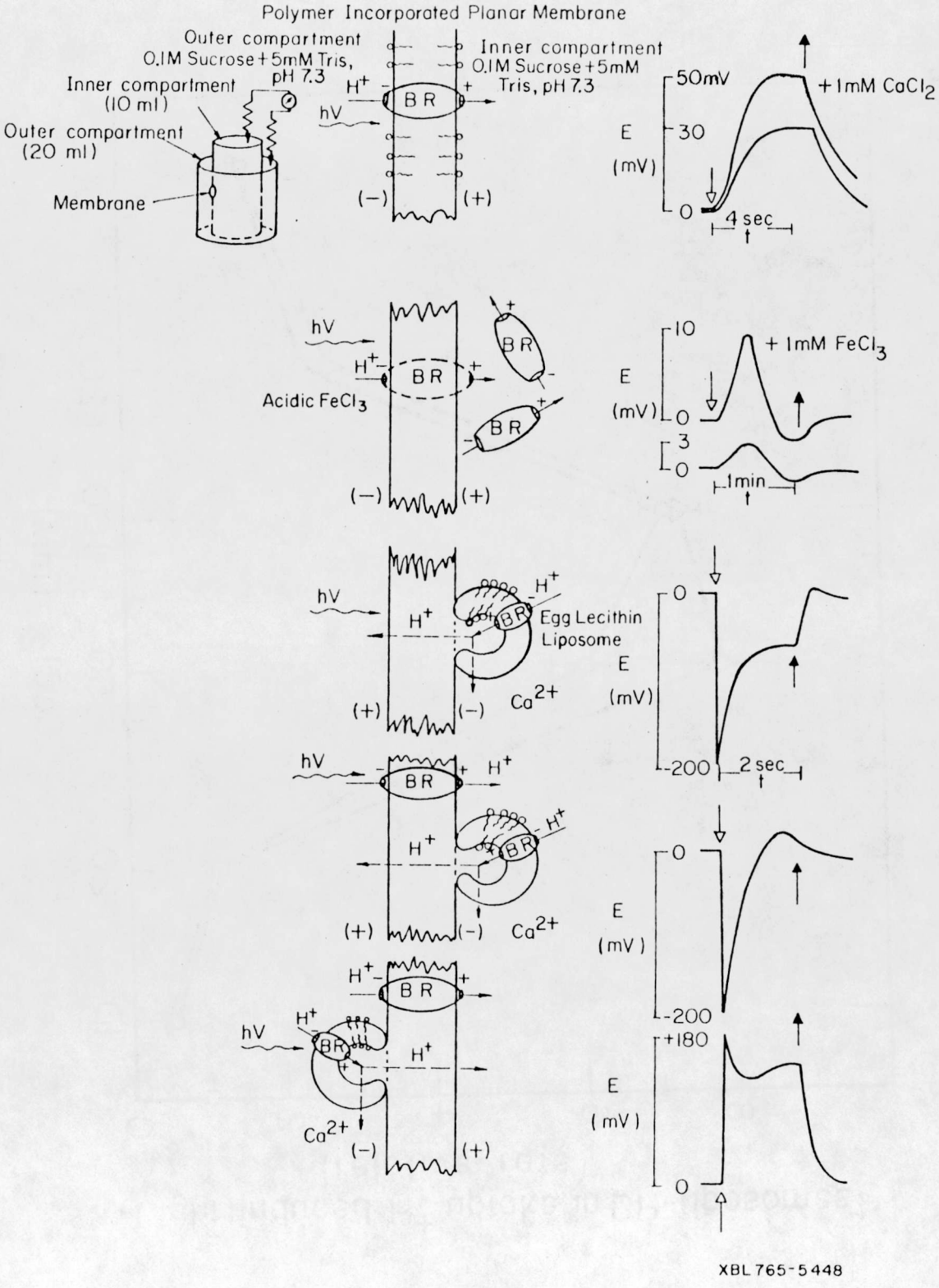
Polymer Incorporated Planar Membrane
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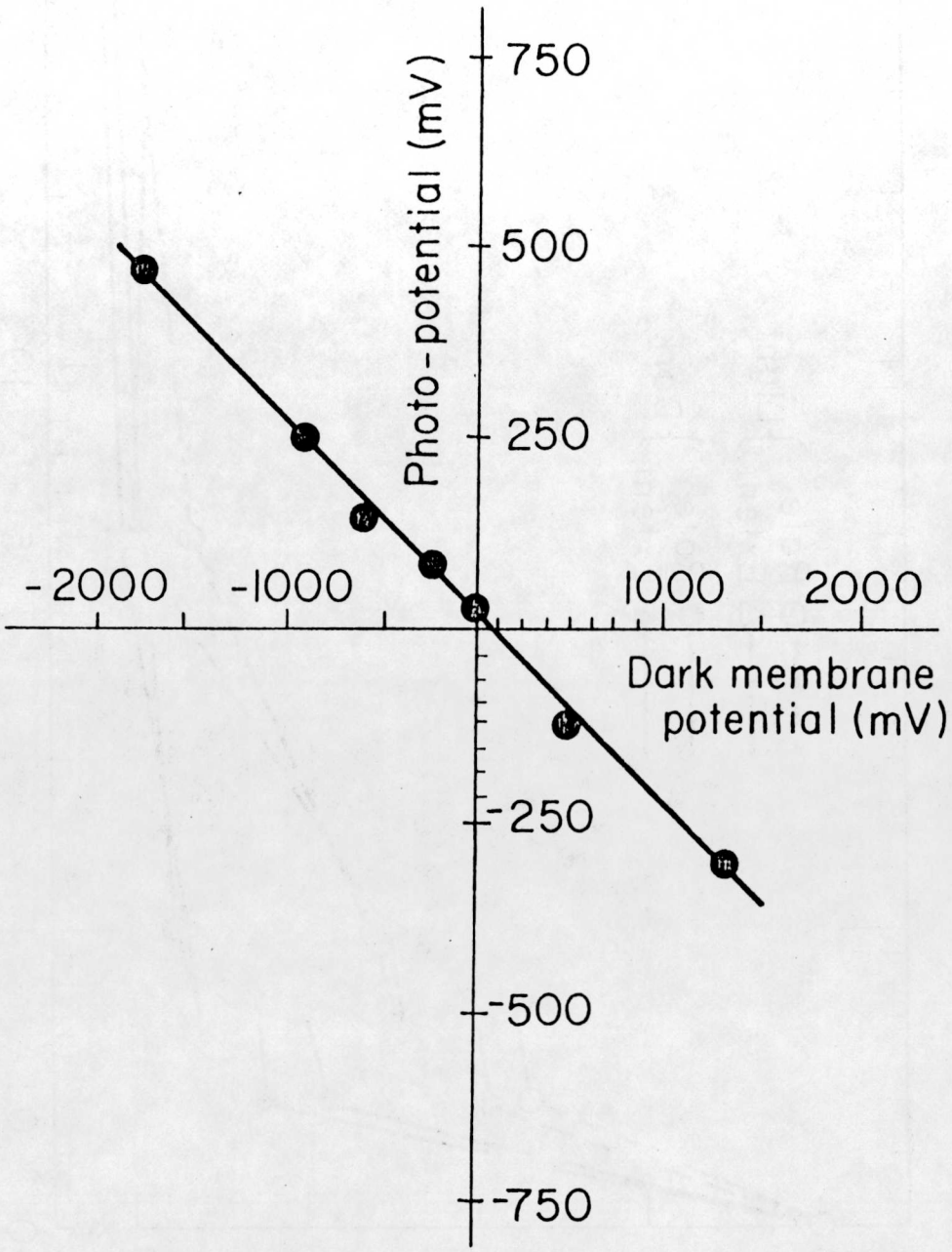
0.1M Sucrose + 5mM Tris, pH 7.3
Inner compartment (10 ml)
Outer compartment (20 ml)
Membrane
Inner compartment
0.1M Sucrose + 5mM Tris, pH 7.3
hV
H+
BR
(−)
(+)
50mV
+1mM CaCl2
E
(mV)
30
0
4 sec
Acidic FeCl3
10
+1mM FeCl3
3
1min
Egg Lecithin Liposome
Ca2+
-200
2 sec
+180
XBL 765-5448


750
500
250
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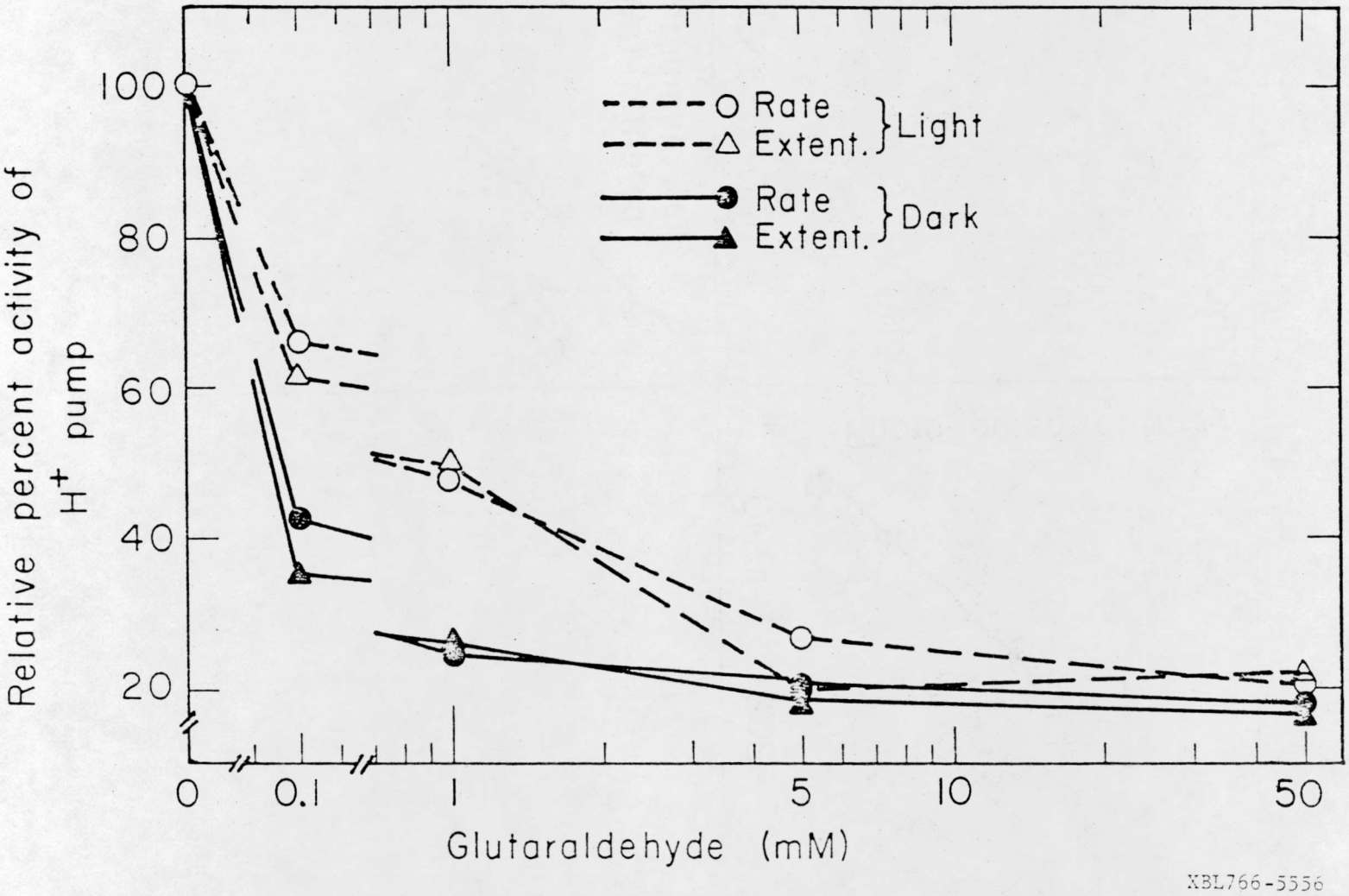
-500
-750
-2000
-1000
1000
2000
Photo-potential (mV)
Dark membrane
potential (mV)

XBL764-5399


Relative percent activity of H+ pump
100
80
60
40
20
0
0.1
1
5
10
50
Glutaraldehyde (mM)
Rate
Extent.
Light
Rate
Extent.
Dark

XBL766-5556

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